



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁴ : C12N 15/00	A1	(11) International Publication Number: WO 87/ 01130 (43) International Publication Date: 26 February 1987 (26.02.87)
<p>(21) International Application Number: PCT/US85/01542</p> <p>(22) International Filing Date: 15 August 1985 (15.08.85)</p> <p>(71) Applicant: STAUFFER CHEMICAL COMPANY [US/US]; Westport, CT 06881 (US).</p> <p>(72) Inventor: MASCARENHAS, Desmond ; 17 Skylark Drive, #31, Larkspur, CA 94939 (US).</p> <p>(74) Agent: LAUDER, Leona, L.; Stauffer Chemical Company, 1200 South 47th Street, Richmond, CA 94804 (US).</p> <p>(81) Designated States: AU, BE (European patent), BR, CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, NL (European patent), NO, SE (European patent), SU.</p>		<p>Published <i>With international search report.</i></p>
<p>(54) Title: TRYPTOPHAN PRODUCING MICROORGANISM</p> <p>(57) Abstract</p> <p><i>Escherichia coli</i> microorganisms carrying plasmid-borne genetic information for the production of L-tryptophan and methods for producing and increasing the fermentative production of L-tryptophan.</p> <div data-bbox="966 1134 1591 1974"> <p>The figure shows three circular plasmid maps. pD 2643 (10.4 kb): Features a central circle with 'pD 2643 (10.4 kb)' and an outer ring with markers: Bgl II (1), Eco RI (4), (ori) (3), Eco RI (2), and Sal I (1). pD 2625 (10.5 kb): Features a central circle with 'pD 2625 (10.5 kb)' and an outer ring with markers: Eco RI (9B), 10, 9C, Sph I (7), Bam HI (9D), Bgl II (4), 5A, 6, Cla I (9A), Eco RV (9F), Bam HI (58), and (ori) (8). pD 2634 (8.4 kb): Features a central circle with 'pD 2634 (8.4 kb)' and an outer ring with markers: 11, Sph I (9F), 7, and Sph I (1).</p> </div>		

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TRYPTOPHAN PRODUCING MICROORGANISMCross reference to Related Applications

This application is a continuation-in-part of application Serial No. 642,303, filed August 20, 1984.

Background of the Invention

The present invention relates to Escherichia coli (E. coli) microorganisms carrying plasmid borne genetic information for the production of L-tryptophan and methods for producing and increasing the fermentative production of L-tryptophan.

Brief Description of Relevant Art

Tryptophan is an amino acid that is an essential component in animal nutrition. Its fermentative production by microorganisms from inexpensive carbohydrate substrates is highly desirable.

10 Fermentative production of tryptophan, using microorganism strains artificially mutated and selected for increased tryptophan production is known. Microorganisms used to this end include Brevibacterium (U.S. Pat. 3,700,539), Enterobacter (U.S. Pat. 4,439,627), Bacillus, particularly Bacillus subtilis [using anthranilate and carbon, nitrogen and
15 mineral sources (U.S. Pat. 4,363,875; Japanese Patent Application J58017190, 1,3-5A, and European Patent Application EP 81-107)] and Corynebacterium glutamicum ATCC 21851.

The application of recombinant DNA methods for the construction of E. coli strains for tryptophan production has been described in a
20 limited way in U.S. Pat. 4,371,614. The E. coli strains described therein carry a specific plasmid having thereon genes of the wild type tryptophan operon (trpA-E) with a wild type or temperature sensitive tryptophan repressor gene (trpR or trpR^{ts}, respectively) for control of tryptophan biosynthesis. Such plasmid or multicopy plasmid according to U.S. Patent
25 4,371,614 may also carry a Serine B gene (SerB). This is particularly desirable when the host strain is a tryptophan or L-serine auxotroph

(i.e., Trp⁻, Ser⁻). Maximum yields disclosed are about 230 ppm of tryptophan. This same patent also describes a number of general methods known to those skilled in the art for carrying out alterations to chromosomal DNA and plasmid DNA, the transformation of microorganism with plasmid DNA, and the selection of microorganism so altered or transformed. U.S. Patent 4,371,614 is hereby incorporated by reference for the purpose of illustrating the general state of the known methods in the field to which the present invention pertains.

A need for inexpensively produced tryptophan and methods for the production of tryptophan as well as methods for increasing yields of tryptophan produced by microorganisms persists.

Objects of the Invention

The present invention is intended to meet the persistent need for inexpensively produced tryptophan by providing a bacterial host microorganism, of the genus Escherichia, deficient in the enzyme tryptophanase carrying plasmid borne information for the production of tryptophan wherein said information is divided between at least two plasmids.

Another object of the invention is to provide a method for producing tryptophan by culturing the above-mentioned bacterial host and plasmids therein in a culture medium and recovering L-tryptophan from the fermented culture medium.

Still another object of the invention is to provide inexpensively produced tryptophan by providing a bacterial host microorganism of the genus Escherichia deficient in the enzyme tryptophanase carrying plasmid borne genetic information for the production of tryptophan wherein a portion of said information for the production of tryptophan is under lac operator/promoter control.

Yet another object of the invention is to provide a method for producing tryptophan by culturing the above-mentioned bacterial host with plasmid borne information for the production of tryptophan, a portion of said information being under lac operator/promoter control in a culture medium and recovering tryptophan from the fermented culture medium.

A still further object of the invention is to increase the production of tryptophan by a bacterial host of the genus Escherichia deficient in the enzyme tryptophanase by transforming said host with plasmid borne information to control tryptophan production divided between at least two plasmids; or by transforming the bacterial host as described with plasmid borne information to control tryptophan production a portion of said information being under lac promoter/operator control, or by transforming said host as described with plasmid borne information to control tryptophan production divided between at least two plasmids, a portion of said information to control tryptophan production being under lac operator/promoter control.

Summary of the Invention

The plasmids used in this invention may be E. coli plasmids or plasmids capable of replicating in E. coli. If the information to control tryptophan production is distributed on more than one plasmid, it is desirable to use plasmids that are replication compatible. Thus, for example, when the information to control tryptophan production is distributed on two plasmids, plasmids having a ColE1 origin of replication may be used to carry part of the information and plasmids having a p15A origin of replication may be used to carry another part of the information.

Useful in the plasmid constructions of the type described in the invention are the following: ColE1, pSC101, pSF2124, pMB3, pMB9, pACYC184, pACYC177, pCK1, R6X, pBR312, pBR313, pBR317, pBR318, pBR320, pBR321, pBR322, pBR333, pBR341, pBR345, pBR350, pBR351, pML2, pML21, ColE1a₇, RSF1010, pVH51, pVH151, pVH153 (Recombinant Molecules: Impact on Science and Society: Beers, R.F., and Bassett, E.G., eds. Raven Press, New York (1977)). Other plasmids are pBR327, pBR325 and pBR328 (Soberon, et al., Gene, 9:287-305 (1980). Plasmid p15A is described in Chang A.C.Y and Cohen, S., J. Bacteriol., 135: 1148-1156 (1978). pHH509 is described in Barth et al., J. Bacteriol., 135:760-765 (1978), and pLG339 is described in Stoker et al., Gene 18:335-341 (1982). Still others are described in "DNA Insertion Elements, Plasmids and Episomes," Bukhari et al. (eds.) Cold Spring Harbor Laboratory (1977). The preferred plasmids are the multicopy plasmids of the type of pBR and its derivatives, ColE1

and its derivatives, p15A and its derivatives, pHH509 and its derivatives and pSC101 and its derivatives.

To ensure equal distribution of the plasmids in daughter cells during growth of the microorganism, it may furthermore be desirable for at least one of the plasmids to contain a par locus (Meacock, P.A. and Cohen, S.N., Cell, 20:529-542 (1980)). If the information to control tryptophan production is distributed among more than two plasmids it is preferred that all plasmids be replication compatible with each other, by constructing them such that they each carry different origins of replication.

The plasmids used in the invention are single copy (stringent) or multicopy (relaxed) plasmids. Because tryptophan yields can be increased by increasing the copy number of plasmids carrying information multicopy plasmids are preferred. Although multicopy plasmids can be frequently lost from the host cell, i.e., are unstable, plasmid copy number may be maintained by constructing the plasmids such that determinants of antibiotic resistance are carried thereon. By adding antibiotics to the culture medium, microorganisms that lose plasmids are eliminated or fall to insignificant numbers, depending on the mode of antibiotic action.

Plasmids used in this invention bear genetic information to control tryptophan production. The term "information to control tryptophan production" according to the invention includes:

1. information controlling the biosynthesis of the common precursors of the aromatic amino acids: tryptophan, tyrosine and phenylalanine;
 2. information for the biosynthesis of tryptophan from chorismic acid via several intermediate steps;
 3. information controlling the biosynthesis of serine which is required in the final step of the tryptophan biosynthetic pathway; and
 4. controlling elements for the information of 1 and 2.
- The controlling elements used in the invention are nucleotide sequences derived from the lactose operon and include the lac operon promoter/operator and lac repressor sequences. According to the invention, various portions of 1 and 2 are under lac control and produce significant amounts

of functional gene products only in the presence of lactose. According to the invention it is preferred that tandem lac operator/promoters are used to achieve greater levels of gene expression than are possible with a single lac promoter.

5 The genetic information to control tryptophan production according to the invention comprises a large number of DNA sequences coding for genes and regulatory sequences thereof, which are plasmid borne. The terms "gene" or "genes" as used herein are meant to encompass a DNA sequence coding for an active product of the gene. Thus, DNA sequences
10 coding for an active gene product are genes within this definition even though the particular DNA sequence may not include the complete structural gene for the active product of the gene.

 In placing this genetic information to control tryptophan production on plasmids it is convenient to divide the genetic information
15 between at least two plasmids. By dividing the genetic information to control tryptophan production between at least two plasmids, the elements can be positioned relative to information they control and their gene products can be produced in sufficient concentration to insure that the information controlling biosynthesis of the common aromatic amino acid
20 precursors, information controlling biosynthesis of tryptophan from chorismic acid and/or information controlling biosynthesis of serine are expressed under optimum conditions and at the desired time in a fermentation cycle. Thus, in a preferred embodiment the plasmid-borne information to control tryptophan production is divided between at least two plasmids.

Brief Description of the Drawing

25 The invention and its advantages over the known prior art can be best appreciated by those skilled in the art from the following detailed description of the invention considered along with the accompanying drawings.

 Figure 1 and Figure 2 are schematic drawings of the preferred
30 embodiments of the plasmids carrying information to control tryptophan production according to the invention. Fragment Numbers 1-11 of Table 1 read in conjunction with Figure 1 identifying each restriction

endonuclease segment of each plasmid. Similarly, Fragment Numbers 12-14 identify the restriction endonuclease segments of the plasmid within Figure 2. Table 1 further identifies each segment by approximate size in kilobases (kb), restriction nuclease ends of each fragment, genes/loci on the fragment, sequence coordinates if known, and journal reference for each known sequence coordinate. The depicted segment sizes in Figure 1 are only approximately proportional to the size of the complete plasmid as shown.

In the drawings, table and in the detailed description of the invention which follows the abbreviations indicated below are used and denote restriction endonuclease enzymes from the following organisms:

<u>Restriction Endonuclease</u>	<u>Source organism</u>
<u>EcoRI</u>	<u>Escherichia coli</u> RY13
<u>BglII</u>	<u>Bacillus globigii</u>
<u>SalI</u>	<u>Streptomyces albis</u> G
<u>PstI</u>	<u>Providencia stuartii</u> 164
<u>Sau3A1</u>	<u>Staphylococcus aureus</u> 3A(1)
<u>SphI</u>	<u>Streptomyces phaeochromogenes</u>
<u>NarI</u>	<u>Nocardia argentinensis</u>
<u>ClaI</u>	<u>Caryophanon latum</u>
<u>AvaI</u>	<u>Anabaena variabilis</u>
<u>BamHI</u>	<u>Bacillus amyloliquifaciens</u> H
<u>XhoI</u>	<u>Xanthomonas holicola</u>
<u>DdeI</u>	<u>Desulfovibrio desulfuricans</u>
<u>NruI</u>	<u>Nocardia rubra</u>
<u>XmnI</u>	<u>Xanthomonas manihotis</u>
<u>MstI</u>	<u>Microcoleus .sp.</u>
<u>NcoI</u>	<u>Nocardia coralina</u>
<u>HindIII</u>	<u>Haemophilus influenzae</u> Rd
<u>AhaIII</u>	<u>Aphanatheca holophytica</u>
<u>SstI</u>	<u>Streptomyces stanford</u>

TABLE 1

Fragment Number	Size (kb)	Flanking Restriction Endonuclease ends		Genes/loci on fragment**	Sequence coordinates, if known	Sequence Reference
		1	2			
1	5.8	BglII'	SalI	trpA, B, C, D	1190-7015	Nucl. Acids Res., 9:6647-6668 (1981)
2	1.6	Eco RI	SalI	trpE _{fbr} (<i>S. marcescens</i>)	22 bp upstream of initiation codon -- 28 bp downstream of termination codon	J. Mol. Biol., 142:503-517 (1980)
3	2.55	Eco RI	Eco RI	tet ^r , ori (<i>ColE1</i>)	1-2143 plus 2854-3274.	Gene, 9:287-305 (1980)
4	0.44	Eco RI	BglII	lacP (UV5) (2 copies)	(a)	Proc. Nat. Acad. Sci., 73: 4174-4178 (1976)
5 A	1.4	DdeI/BglII	XmnI*	aroG _{fbr}	476-1864	Nucl. Acids Res., 10:4045- 4058 (1982)
B	0.95	Clal	Sau3A/BamHI		1096-2058	
6	1.7	NruI*	EcoRV	serA	from 98 bp upstream of initiation codon to 370 bp downstream of termination codon	(D.M., unpublished)
7	1.1	SphI	NarI*	lacI	100 bp upstream of initiation codon to bp 1020 of lacI sequence	Nature, 274: 765-769 (1978)
8	2.6	AvaI/BamHI	Clal*	cam ^R , ori (P15A)	(b)	J. Bacteriol., 134:1141- 1156 (1978)

TABLE 1
(continued)

Fragment Number	Size (kb)	Flanking Restriction Endonuclease ends		Genes/loci on fragment**	Sequence coordinates, if known	Sequence Reference
		1	2			
9 A	0.16	<u>Clai</u>	<u>Eco RV</u>	-	24-187	<u>CSH Symp. Quant. Biol., 43: 77-90 (1979)</u>
B	0.22	<u>NruI</u> / <u>Eco RI</u>	<u>NarI</u> *	-	973-1205	
C	0.86	<u>SphI</u>	<u>AvaI</u>	-	565-1424	
D	0.35	<u>Clai</u> *	<u>BamHI</u>	-	24-375	
E	0.75	<u>PstI</u> / <u>BamHI</u>	<u>Eco RI</u>	-	3613-4363	
F	0.54	<u>Clai</u> *	<u>SphI</u>	-	24-565	
10	0.38	<u>Eco RI</u>	<u>AvaI</u>	<u>par</u> (pSC101)	1-375	<u>Gene, 24:309-315 (1983)</u>
11	6.8	<u>SphI</u>	<u>SphI</u>	<u>amp^r, ori</u> (pHH509)	(c)	<u>J. Bacteriol., 135:760-765 (1978)</u>
12	5.9	<u>BamHI</u>	<u>SalI</u> *	<u>Kan^r, ori</u>		
13	5.3	<u>XhoI</u> *	<u>BglII</u>	<u>serC</u>		
14	4.0	<u>BglII</u>	<u>BamHI</u>	<u>serB</u>		

(a) Each lacP segment (there are 2 in tandem) consists of the 203 bp HaeIII fragment which spans this promoter in E. coli. Downstream of these promoters is a 35 bp sequence (from the cro gene of phage lambda) terminating in a BglII site.

(b) 2.6 kb AvaI-Clai fragment of pACYC184.

(c) A derivative of pHH509 was constructed by in vitro deletion of a 0.75.kb PstI fragment. This derivative was then digested with SphI to yield a 6.8 kb fragment.

▲ Conversion of terminii in vitro.

* Destroyed at junction with adjacent fragment.

** All E. coli genes unless otherwise specified.

Experimental Methods

In the construction of the host strains and plasmids according to the invention, a number of methods known to those skilled in the art are employed. These methods are substantially described in numerous journals and those cited herein are well known examples of such method
5 descriptions.

Transduction using P1 phage lysates is described in Rosner, J.L., Virology, 49:679-689 (1972). The use of Tn10 transposable elements for insertion into and deletion of chromosomal regions is described in Kleckner, N. et al., Proc. Nat. Acad. Sci., USA 73:3838-3842 (1976).
10 Transformation of host strains with the plasmids according to the invention is carried out by calcium shock of the host strain rendering the host capable of incorporating DNA as described, for example, in Morrison, D.A. et al., J. Bacteriol., 132:342-351 (1977). Alternatively, E. coli host strains may be transformed by growing them to an optical density of about
15 0.6 at 600 nanometers in liquid medium, chilling the cell suspension on ice for approximately 5 minutes, harvesting the cells by centrifugation, washing the cells in chilled 10 mM MgCl₂, harvesting the cells, resuspending in chilled 50 mM CaCl₂ for about 30 minutes and adding plasmid DNA in suspension and maintaining the mixture at 0°C for about 45 minutes; the
20 cells are resuspended in growth medium for about one hour at 37 °C. Transformed host cells are selected or screened on appropriate medium.

The λ 1059 Sau3A E. coli genomic DNA clone bank was constructed by ligating Sau3A partial digests of E. coli genomic DNA to BamHI-cleaved λ 1059 arms, thus generating a bank of recombinant phage containing ran-
25 dom pieces of the entire E. coli genome. (Karn et al., Proc. Nat'l. Acad. Sci., USA vol. 77 9:5172-5176 (1980).) The general methods for generating such a λ 1059 Sau3A E. coli genomic DNA clone bank are well known to those skilled in the art. Clone banks using other vectors and restriction endonuclease digests are also known.

30 Plasmids are generally isolated from host cells by the Birnboim cesium chloride gradient method. (Birnboim, H.C., Doly, J., Nucleic Acids Research, 7:1513-1523 (1979).)

After restriction endonuclease digestion, plasmid fractions are generally fractionated by electrophoresis on an agarose or acrylamide gel, with appropriate molecular weight standards, using a buffer of 4 mM Tris, 1 mM EDTA adjusted to pH 8.2. Fragments are subsequently washed in ethanol and resuspended in Tris EDTA buffer at pH 8.0.

Ligation of restriction endonuclease-cut DNA fragments is generally carried out with T₄ ligase in 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 20 mM dithiothreitol, 1 mM ATP and 50 ug/ml bovine serum albumin. T₄ ligase may be used to catalyze the formation of phosphodiester bonds between juxtaposed 5' phosphate and 3'-hydroxyl termini in double-stranded DNA. Both blunt and cohesive end restriction fragments may be ligated using T₄ ligase.

Unpaired terminal bases of restriction endonuclease cut DNA fragments are made blunt-ended with DNA polymerase I (PolI) in the presence of deoxyribonucleotides (dNTP's). In general, approximately 1 microgram (ug) of the purified DNA fragment having unpaired terminal bases is incubated with 2 units of Klenow DNA Polymerase I (Jacobsen et al., Eur. J. Biochem., 45:623-627 (1974)), 10 mM β -mercaptoethanol, 6 mM Tris-HCl (pH 7.5), 8 mM magnesium chloride and 0.2 mM each of the following dNTP's: deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), thymidine triphosphate (TTP) and deoxyguanosine triphosphate (dGTP). The reaction is run at 16°C for 60 minutes and is terminated by heat inactivation at 65°C for 10 minutes followed by rapid cooling on ice.

A number of strains used in making the host strain according to the invention were obtained from the E. coli Genetic Stock Center (CGSC) Department of Human Genetics, Yale University School of Medicine, P.O. Box 3333, Cedar Street, New Haven, Connecticut 06510, USA. Strains obtained from the CGSC are designated herein by a researcher's name "via B. Bachmann", curator for CGSC.

Media for antibiotic selection or screening of plasmid containing microorganisms is formulated to sustain growth of the microorganism of interest thereon, but contains, unless otherwise indicated, 20 mg/l of the antibiotic of interest.

Media used in the construction of the host strains and plasmid described hereinbelow are as follows:

LB per liter

tryptone 10 grams (g)
yeast extract 5 g
sodium chloride 5 g
glucose 2 g

LB plates = LB + 1.5% agar
LB Mg = LB + 0.01M $MgCl_2$
LB Ca = LB + 0.005M $CaCl_2$
LB Top Agar = LB + 0.75% agar
Saline = 0.85% NaCl

Fusaric Acid Plates

agar 15 g
Difco® tryptone 10 g
Difco® yeast extract 5 g
sodium chloride 10 g
glucose 2 g
chlortetracycline 50 milligrams (mg)
 $NaH_2PO_4 \cdot H_2O$ 10 g

autoclave, cool, then add
6 ml 2 mg/ml fusaric acid
5 ml zinc chloride 20 mM

Minimal Media per liter

citric acid 2 g
80% H_3PO_4 4.9 g
KCl 4 g
30% NH_3 solution 5.86 g
 $FeSO_4 \cdot 7H_2O$ 1 mg
 $MnSO_4$ 1 mg
 $MgSO_4 \cdot 7H_2O$ 1 g
thiamine-HCl 3.5 mg

Supplements to Minimal Plates

amino acids 20 micromoles/milliliter (ug/ml)

tetracycline 10 ug/ml

-2-thienyl-DL-alanine 20 ug/ml

Difco® casamino acids 0.4%

3-fluoro tryptophan (20 ug/ml)

7-methyl tryptophan (20 ug/ml)

biotin 1 ug/ml

thiamine 1 ug/ml

triphenyltetrazolium chloride (20 ug/ml)

Vogel-Bonner Minimal Medium (per liter)

MgSO₄·7H₂O 0.2 g

citric acid 2 g

K₂HPO₄ 10 g

NaNH₄PO₄·4H₂O 3.5 g

Make 0.4% glucose after autoclaving. Plates (solid medium) are made by adding agar (1.5%).

CONSTRUCTION OF SPECIFIC PLASMIDSConstruction of Plasmid pD2643

Plasmid pD2643 comprising trpA-E genes of which trpA-D are under lac promoter control, lacking a trp attenuator region, carrying a tetracycline resistance gene (tet^r) and having a ColE1-type origin of replication was constructed as follows.

- 5 Tandem lac promoters were removed from pKB252 (lac PUV5) (K. Backman et al., Proc. Nat'l. Acad. Sci. USA, 73:4124-4178 (1976)) by partial digestion with EcoRI and complete digestion with SalI restriction endonucleases under buffer conditions suggested by the manufacturer, and were ligated using T₄ ligase into plasmid pBR327 which had been previously
10 digested to completion by SalI and EcoRI. After transforming E. coli, tetracycline resistant (Tet^r) ampicillin resistant (Amp^r) colonies were isolated. The resident plasmid in one of these was designated pDM126.

- pDM126 was partially digested with BglII and completely digested with SalI restriction endonucleases. A 5.8 kilobase (kb) BglII-SalI fragment derived from pDB107 (Mascarenhas et al., Virology, 24:100-108 (1983))
15 carrying the trpD, C, B, A genes and lacking most of trpE gene as well as

the attenuator and promoter regions of the trp operon (corresponding to nucleotides 1190-7015 identified by Yanofsky et al., Nucl. Acids Res., 9:6647-6668 (1981)) was ligated using T₄ ligase into the BglIII, SalI-digested pDM126 forming a mixture of ligated DNA. Plasmid pDM136, having 5 trpA-D under lac control was identified by transforming strain AE2 (Benedik et al., Virology, 126:653-668 (1983)), which lacks trpA-E, with the ligated DNA and selecting transformed microorganisms on minimal medium containing ampicillin and anthranilate.

A SalI-EcoRI fragment containing a gene for anthranilate synthetase resistant to feed-back inhibition by tryptophan was obtained as follows. An EcoRI digest of Serratia marcescens genomic DNA was prepared, and from an agarose gel a 4-9 kb fraction thereof was isolated. Said 4-9 kb fraction was ligated into EcoRI-digested pACYC184 under conditions favoring formation of hybrid molecules, and the resultant plasmid was used 15 to transform E. coli strain MV17 (D. Helsinki) deleted for trpE. Colonies were selected that grow on minimal medium. Plasmid pC501 carrying trpC-E was isolated from one of the selected colonies.

Plasmid-linked 7-methyltryptophan (7 MT) resistance was selected by treating a strain carrying pC501 with ethyl methane sulfonate and 20 selecting for resistant mutants on minimal medium containing 7MT and tetracycline. One such mutant had a plasmid gene (trpE) coding for an anthranilate synthetase resistant to inhibition by tryptophan.

A 1.6 kb SalI-EcoRI fragment of the mutant derivative of pC501, having an altered trpE gene encoding an anthranilate synthetase resistant 25 to inhibition by tryptophan was ligated into SalI-PstI digested pDM136. An EcoRI-PstI fragment of pBR327 containing a tet^r gene was added to this ligation mixture. Plasmid pD2623 coding for the activities of the trpA-D genes under lac control and for trpE (feedback resistant anthranilate synthetase) and which specifies Tet^r and Amp^r was identified by transforming 30 strain MV17 which is a tryptophan auxotroph, selecting for Tet^r-Amp^r and screening these colonies for growth in the absence of tryptophan (i.e., tryptophan independence) on minimal medium. Plasmid pD2643 was constructed by deleting Amp^r activity from pD2623 by digestion with AhaIII restriction endonuclease, ligation with the T₄ ligase, transformation of

an E. coli host microorganism to Tet^r and screening for ampicillin sensitivity.

Construction of Plasmid pD2625

Plasmid pD2625 coding for aroG activity which is feedback resistant to inhibition by aromatic amino acids (under lac control), serA and lacI activity, a par locus and a p15A origin of replication was constructed in the manner described immediately below.

A. Construction of serA-Containing Donor Plasmids

A nucleotide sequence (λ 1059 serA) derived from an E. coli K12 genomic clone bank (Karn et al., Proc. Nat'l. Acad. Sci., 77, 9:5172-5176 (1980); clone bank obtained from M. Benedik, Stanford University) was isolated, digested with NcoI restriction endonuclease and ligated into NcoI-digested pACYC184 under conditions favoring formation of hybrid molecules. E. coli serine auxotroph JC158 (A.J. Clark via B. Bachmann) was transformed to Tet^r and serine independence using aliquots of the hybrid molecules. pD2528 was isolated from a serine independent Tet^r transformant of JC158. pD2528 DNA was digested with EcoRI and SalI and was ligated using T₄ ligase into EcoRI-SalI digested pBR327 under conditions favorable for plasmid formation. pD2537 was isolated from transformants of E. coli (JC158) that were Amp^r, Tet^s and Ser⁺.

B. Construction of lacI Activity Donor Plasmids

A bacteriophage carrying the genes of the lac operon was obtained from the λ 1059-E. coli genomic bank indicated above. Lac activity was identified by complementation of a Lac⁻ E. coli strain (B1361) carrying plasmid pDE4081 (Mascarenhas et al. Virology, 26:658-668 (1983)) on lactose minimal plates. The λ 1059 lac phage thus selected was digested with BglII. A 10.5 kb BglII fragment obtained from this digest was ligated to BamHI-digested pACYC184 and the hybrid was used to transform B1361 to Cam^r Lac⁺. A 4.2 kb EcoRI-SphI sub-fragment of this plasmid was then ligated to a 2.7 kb EcoRI-SphI fragment previously obtained from pBR327, to form pD2329. Finally, digestion of pD2329 with NarI restriction endonuclease yielded a 1.85 kb NarI fragment with lacI sequences from about 100 base pairs upstream of the lacI promoter to base pair number 1020 in the lacI DNA sequence reported by Farabaugh in Nature,

274:765-769 (1978). This 1.85 kb NarI fragment was ligated into pACYC184 digested with ClaI restriction endonuclease-forming plasmid pD2333 having lacI activity, Cam^r and a P15A origin from plasmid pACYC184. A SalI restriction site located between an AvaI and BamHI site on plasmid pD2333
5 was eliminated by digestion with AvaI followed by addition of Klenow Pol I, dNTP's, BamHI linkers and T₄ ligase. This was followed by digestion with BamHI and re-circularization of the resulting large fragment with T₄ ligase, to form pD2348.

Plasmid pD2348 was digested with restriction endonucleases NruI
10 and SalI. The digest was combined with a 1.1 kb EcoRI-SalI fragment of pPM31 containing a partition (par) locus (Meacock and Cohen, Cell, 20:529-542 (1980)), and T₄ ligase, Klenow PolI and dNTP's. The resulting plasmid pD2349 which is Cam^r and has a P15A origin of replication, par locus and lacI activity was isolated by screening for Cam^r transformants.

C. Construction of aroG^{fbr} Donor Plasmid

15 A bacteriophage carrying the aroG gene of E. coli was selected from the λ 1059-E. coli genomic bank indicated above by complementation of an Aro⁻ (aroG) strain, C531, described below, carrying plasmid pDE4081. A 5.8 kb BamHI-SalI fragment of DNA from this bacteriophage was subcloned into BamHI-SalI digested pACYC184 to form pC520.

Construction of pD2625 From Donor Plasmids

20 Plasmid pC520 was digested with BglII and BamHI restriction endonucleases, and a 1.6 kb BglII-BamHI fragment from this digest was ligated into BglII-BamHI digested plasmid pDM126 using T₄ ligase to form pD2149, a plasmid in which aroG activity is under tandem lac promoter transcriptional control.

25 pD2149 was digested with PstI to form PstI fragments. These fragments were ligated to a BamHI adaptor sequence which converts the PstI fragments ends to BamHI ends. From the converted BamHI fragments, a 2.8 kb BamHI fragment carrying the aroG gene under tandem lac promoter control was isolated by gel electrophoresis and was ligated using T₄ ligase into
30 BamHI digested pD2349 to form pD2351 which is characterized as follows:

Cam^r, p15A origin, par locus, lacI activity, tandem lac promoters linked to aroG.

A 2-nucleotide shift in the aroG locus of pD2351 was introduced by ClaI restriction endonuclease digestion in the presence of Klenow PolI and dNTP's, followed by treatment with T4 ligase. The frame shift renders pD2351 incapable of expressing DAHP synthetase, the peptide product of aroG, yet conserves practically the whole aroG nucleotide sequence. The plasmid with the frame shift is used to transform E. coli strain MAR13 (Held and Smith, J. Bacteriol., 101:202-208 (1970)), a strain carrying chromosomal aroG activity which is feedback resistant to inhibition by phenylalanine and hence resistant to aromatic amino acid inhibition. In this transformant, low frequency homologous exchange of DNA between the Mar13 chromosomal aroG and the plasmid pD2351 (frame-shifted) aroG gene occurs. Plasmid DNA is isolated from this strain by conventional methods and is used to transform E. coli strain C531 which is a spontaneous Gal^rTet^r derivative of NK6969 (Roberts, D.) and carries a complete deletion of the aroG gene. Colonies are selected for growth on minimal medium containing nicotinamide, chloramphenicol, tyrosine and tryptophan. Colonies that grow under these conditions are screened individually by enzyme assay [Doy and Brown, Biochemica et Biophysica. Acta., 104:377389 (1965)] to identify those which contain a feedback resistant DAHP synthetase (aroG^{fb^r}) activity. The plasmid in one such isolate was designated pD2422.

Plasmid pD2434 in which the aroG^{fb^r} activity is brought under lac operator control is constructed from pD2422 as follows: a sample of plasmid pD2422 is digested with EglII restriction endonuclease, Klenow Pol I and dNTP's to form an 8.5 kb fragment having blunt ends. The 8.5 kb blunt-ended fragment is digested with XhoI restriction endonuclease to form a 7.4 kb fragment having one blunt end and one XhoI end.

Another sample of pD2422 is digested with DdeI restriction endonuclease and Klenow Pol I with dNTP's to form multiple fragments with blunt ends. These blunt ended DdeI fragments are then digested with XhoI restriction endonuclease to form a 1.0 kb fragment having an XhoI end and

a blunt end. The 7.4 kb and 1.0 kb fragments are purified, mixed and ligated using T₄ ligase, to form plasmid pD2434.

Plasmid pD2625 was constructed from pD2434 and pD2537 in the following manner. pD2537 was digested with ClaI and NruI restriction endonuclease to yield a 1.85 kb fragment having a ClaI end and a blunt end. A sample of pD2434 was digested with ClaI and BglII restriction endonuclease to yield a 7.7 kb fragment with a BglII site adjacent to and downstream of the lac promoter and a ClaI site in the aroGfbr gene.

A second sample of pD2434 was digested with XmnI and BglII restriction endonuclease to yield a 1.4 kb fragment having an XmnI end and a BglII end. The 7.7 kb fragment of pD2434, 1.85 kb fragment of pD2537 and 1.4 kb fragment of pD2434 were ligated together with T₄ ligase and were used to transform JC158 (J.C. Clark via B. Bachmann) to Cam^rSer⁺. One of these transformants yielded plasmid pD2625.

A plasmid pD2624 in which the serA fragment is inserted into pD2434 in the opposite reading sense from that of pD2625 was also constructed by digesting a sample of pD2434 with XhoI and BglII to yield a 7.4 kb fragment having XhoI and BglII ends, and digesting a second sample of pD2434 with XhoI and BglII as before. Plasmid pD2537 was digested with SalI and MstI to yield a 2.0 kb fragment with a SalI end and MstI blunt end. The fragments were ligated together with T₄ ligase as above. The SalI end of the serA-containing fragment from pD2537 and XhoI end of the large pD2434 fragment are compatible as these two endonucleases produce compatible cohesive termini.

Construction of Plasmid pGM3207

Plasmid pGM3207 (shown in Figure 2) carrying serB and serC was constructed in the manner described immediately below in Sections A-C.

A. Construction of serB-Containing Donor Plasmid

An E. coli serine auxotroph was constructed by transducing strain CGSC #5409 (argI61, argF58, serB28, purA54, thr-25, tonA49, relA1, spoT1; obtained from B. Bachmann) to Pur⁺ with P1 phage grown on strain W3110. One Pur⁺ transductant was purified and designated G3004.

A nucleotide sequence (λ 1059 serB) derived from an E. coli K12 genomic clone bank (Karn et al., Proc. Nat'l. Acad. Sci., 77, 9:5172-5176 (1980); clone bank obtained from M. Benedik, Stanford University) was isolated, digested with BamHI restriction endonuclease and ligated into
5 BamHI-digested pLG339 under conditions favoring the formation of hybrid molecules. E. coli serine auxotroph G3004 was then transformed to Kan^r and serine independence using aliquots of the hybrid molecules. Plasmid pGM3147 was isolated as a serine independent Kan^r, tet^S transformant of G3004.

B. Construction of serC-Containing Donor Plasmid

10 A nucleotide sequence (λ 1059 serC) derived from an E. coli K12 genomic clone bank (supra) was isolated, digested with NcoI restriction endonuclease and ligated into NcoI-digested pACYC184 under conditions favoring formation of hybrid molecules. E. coli serine auxotroph CGSC #4297 (K.B. Low via B. Bachmann) was transformed to tet^r and serine
15 independence using aliquots of the hybrid molecules. Plasmid pGM3134 was isolated as a serine independent tet^r transformant of CGSC #4297.

C. Construction of pGM3207 From Donor Plasmids

Plasmid pGM3134 was digested with BglII and XhoI restriction endonucleases, to yield a 5.3 kb BglII-XhoI fragment containing serC.

A sample of plasmid pGM3147 was digested with BglII and SalI
20 restriction endonucleases to yield a 9.9 kb BglII-SalI fragment containing serB and a gene specifying kanamycin resistance.

The pGM3134 5.3 kb BglII-XhoI fragment was ligated to the pGM3147 9.9 kb BglII-SalI fragment with T₄ ligase. The products of this ligation mixture were used to transform CGSC #4297 (serC auxotroph) to Kan^r,
25 Ser⁺. Plasmid DNA purified from one of these transformants was used to transform G3004 (CGSC #5409 derivative and serB auxotroph, see above in Section A) to Kan^r, Ser⁺. The transforming plasmid was designated pGM3207.

Construction of Host Strain

B1238 [W3110 F⁻ (argF-lac) Δ U169, Δ (gal-bio), \emptyset (trp-lac) W205, \emptyset (trp- Δ 61-intc-226), trp^R] (Benedik et al., Gene, 19:303-311 (1982)) was transduced to Gal⁺ with P1 phage grown on strain W3110. The resulting strain designated B1238GB was subjected to ethyl methane sulfonate mutagenesis and was plated on glucose minimal medium containing 2000 mg/l anthranilate. One anthranilate resistant colony was selected and was designated A103.

A103 was transduced to tetracycline resistance with a P1 lysate of E. coli strain TST 1 (T.J. Silhavy via B. Bachmann). TST 1 is known to carry a Tn10 insertion in the malE gene (malE52:Tn10) which is closely linked to the gene coding for phosphoglucose isomerase (pgi). Tet^R colonies were selected on LB agar medium with 10 mg/l tetracycline and one of these was designated A103T. A number of spontaneously-occurring fusaric acid resistant derivatives of A103T were isolated on fusaric acid plates. Fusaric acid resistant isolates were screened on glucose tetrazolium plates and those isolates which formed white colonies on this medium were tested further. One of these was found to be auxotrophic for lysine in the presence of methionine and threonine. Since the genes lysC and malE flank the pgi gene it was assumed that the lysine auxotroph had a complete deletion of the pgi locus. This isolate was designated CT10 and was assumed to be (lysC-pgi-malE) Δ .

A derivative of CT10 which was additionally deficient in the activity of tryptophanase (tna), was made by P1 transduction of CT10 to Sal⁺ (growth on salicin plates) with lysates of strain C537. These transductants were screened for tryptophanase activity and one Tna⁻ isolate was selected and named C536. C537 mentioned above had been constructed by P1 transduction of MV17 (D. Helinski) to Sal⁺ using lysates of E. coli strain N1624 (M. Gottesman via B. Bachmann) and selecting one transductant that was still Tna⁻. C534, a Tna⁻ derivative of A103, was constructed in a manner analogous to that described above for C536.

Strain D2307, which carries a mutation in the tyrR gene coding for tyrosine repressor which controls the production of shikimate kinase, was made by P1 transduction of E. coli strain JP2144F (A.J. Pittard via B.

Bachmann) to Trp⁺ with lysates of E. coli strain W3110. D2307 was picked as a 3-fluorotyrosine resistant strain on minimal medium containing 3-fluorotyrosine.

Strain D2316, a strain deficient in the production of phenyl-
5 alanine caused by a deficiency in the pheA gene which codes for the first enzyme of the phenylalanine biosynthetic pathway, was made by conjugation of strain C536 and strain KA197 (H. Okestra via B. Bachmann). D2316 was selected as a Trp⁺ phenylalanine auxotrophic transconjugant by replica plating on medium either containing or lacking phenylalanine.

Strain D2139 was made by P1 transduction of strain MV17 (D.
10 Helsinki, supra) with lysates of MB82 (Benedik et al. Virology, 126:658-668 (1983)). Strain D2316 was made Tet^r by P1 transduction with lysates of strain D2139, which contains a Tn10 insertion in the trpB gene (trpB::Tn10). A Tet^r Trp⁻ transductant was isolated and designated D2317.
15 D2317 was P1 transduced to 3-fluorotyrosine resistance with lysates of D2307 described above. D2318 characterized as tryptophan independent, 3-fluorotyrosine resistant and phenylalanine-requiring was one of the transductants obtained.

D2318 was P1 transduced using lysates of strain AT2471 (A.L.
20 Taylor via B. Bachmann), a tyrA mutant strain. Phenylalanine-independent colonies were selected by growth on medium containing tryosine but lacking phenylalanine. Phenylalanine-independent colonies were screened for tyrosine dependence and a Phe⁺Tyr⁻ strain was isolated and designated D2320.

25 A strain having feedback resistant anthranilate synthetase was constructed from strain D2320 by first making D2320 Tet^r by P1 transduction with lysates of D2319. The trpB::Tn10 sequence was introduced into D2320 in a manner analogous to that used in making D2317. One Tet^r transductant (D2324) was P1-transduced using lysates of a spontaneously occurring
30 derivative of W3110 having anthranilate synthetase resistant to feedback inhibition by tryptophan. A tryptophan independent, 7-methyltryptophan-resistant transductant that was auxotrophic for tyrosine and sensitive to tetracycline was selected and designated D2325. Enzyme assays (according

to Ito and Crawford, Genetics, 52:1303-1316 (1965)) in the presence of 2 mM L-tryptophan confirmed that D2325 specified a feedback-resistant anthranilate synthetase.

A strain designated D2327 which is auxotrophic for phenylalanine and tyrosine independent was made by P1 transduction of D2325 to Tyr⁺ using lysates of strain KA197. (Hoekstra via B. Bachmann).

Strain D2327 was made Tet^r by P1 transduction with lysates of an E. coli strain (C541) having a Tn10 insertion in the nadA locus and an adjacent deletion removing nadA, aroG, gal. C541 was made by P1 transduction of MV17 (D. Helsinki, supra) with lysates of strain C531. The Tet^r derivative of D2327 was then transduced using P1 lysates of strain MAR13 (Held and Smith, supra). Mar13 carries an aromatic amino acid feedback resistant DAHP synthetase activity. Transduced cells were isolated as Nad⁺, Phe⁻, tetracycline sensitive strains. In one transductant, feedback resistance of DAHP synthetase to phenylalanine was determined by enzyme assay in the presence of 1 mM phenylalanine. This strain was designated D2346. A phenylalanine independent strain of D2346 was made by P1 transduction of D2346 using lysates of AT2471 (A.L. Taylor, supra) and selecting for phenylalanine independence on medium containing tyrosine. One Phe⁺Tyr⁻ transductant was designated D2402.

B1364 is a trp⁺lac⁺ transductant of B1363 (Mascarenhas et al., Virology, 124:100-108, (1983)) using P1 lysates of W3110. B1364 thus has genetic information for β -galactosidase, lactose transacetylase, and lactose permease activity all fused to and under the control of the tryptophan operon (ϕ trp-lac W205). Strain D2402 was made Tet^r by P1 transduction using a lysate of D2139 as above; a Tet^r derivative of D2402 was then transduced to Trp⁺Lac⁺ with P1 lysates of strain B1364. One Trp⁺Lac⁺Tet^s 7-methyl-tryptophan resistant (7MT^r) isolate was designated D2432.

D2432 was made Tet^r by P1 transduction using P1 lysates of strain RS162 (J. Wechsler via B. Bachmann). The resulting Tet^r strain (D2548) has a Tn10 insertion in the zjb locus and a temperature sensitive mutation dnaB252. A spontaneous isolate of this strain capable of

utilizing hydroxyphenyl pyruvate was isolated and designated D2549. D2549 was P1-transduced using lysates of strain DG30 (D. Gelfand via B. Bachmann), a strain lacking transaminase A (tyrB⁻). Transductants were isolated by growth on LB agar at 42°C (loss of dnaB252 mutation) and
5 screened for inability to grow on plates containing p-hydroxyphenylpyruvate. One such isolate was designated D2550.

A spontaneous tetracycline sensitive (fusaric acid resistant) derivative of D2550 designated D2618 was selected on fusaric acid plates. D2618 was then made Tet^r by P1 transduction using lysates of NK6024 (N. Kleckner via B. Bachmann), a strain having a Tn10 insertion in the pheA locus. Tetracycline resistant colonies were selected and one Phe⁻Tyr⁻ transductant was designated D2636. Spontaneous fusaric acid resistant (Tet^S) derivatives of D2636 were selected on fusaric acid plates and checked for phenylalanine and tyrosine requirements. One such isolate
15 (Phe⁻Tyr⁻Tet^S) was designated D2637.

D2637 was transduced to Tet^r (Ser⁻) using P1 lysates of strain 123A1, which contain a Tn10 insertion linked to the serC locus in strain strain KL282 (K. B. Low via B. Bachmann). One Tet^rSer⁻ transductant, designated D2638, was transduced to Ser⁺ with P1 lysates from DG30 (D. Gelfand via B. Bachmann) which contains an aspC mutation and is auxo-
20 trophic for aspartic acid. These transductants were screened for aspartic acid auxotrophy. One aspartic acid auxotroph that was serine independent and Tet^S was designated D2639.

Serine deaminase-deficient derivatives of D2637 were constructed
25 as follows: Strain MEW191 and a P1-sensitive derivative of strain 1K15-5 (both strains received from E. Newman, Concordia University) are believed to carry mudX (Cam,Amp) insertions in regulatory and structural genes for L-serine deaminase, respectively. A P1 lysate of each of these strains was used to transduce D2637 to Cam^rAmp^r. Single transductants from each
30 cross were designated D2711 and D2713 respectively, and were deemed to carry the mudX (Cam, Amp) insertions (regulatory:D2711, structural:D2713) present in their respective parents.

Spontaneous Cam^SAmp^S derivatives of D2711 and D2713 were isolated and designated D2714 and D2715 respectively. Enzyme assays confirmed that both D2714 and D2715 were deficient in L-serine deaminase activity.

Improved Tryptophan production in Pgi⁻ host compared to Isogenic Pgi⁺ host

C534 and C536 are isogenic E. coli strains except that the pgi gene has been deleted in C536. C534 was made by P1 transduction of A103 using lysates grown on C537 and screening for Tna⁻ colonies. C536 was described above. These strains were cured of their resident plasmids by isolation of Amp^S segregants in each case. These cured derivatives were then transduced to Tet^R with P₁ grown on D2636 (tyrA4 pheA18::Tn10).
Single transductants were then cured of the transposon by selection of spontaneous fusaric-acid-resistant segregants on fusaric acid plates. One of these, D2704, was Pgi⁺Tyr⁻Phe⁻Tet^S (derived from C534). D2705, which is Pgi⁻, was derived from C536 in a way similar to D2704 and is otherwise isogenic to D2704.

D2704 and D2705 were each transformed sequentially with pD2634, pD2643 and pD2625 - by selection for Amp^R, Tet^R and Cam^R, respectively, using conventional calcium shock to render the host competent for transformation. These plasmids carry the genes for the key enzymes of tryptophan biosynthesis and were described hereinabove. One triple transformant in each case was used for nuclear magnetic resonance (NMR) analysis.

¹³C-glucose NMR spectroscopy of D2704 and D2705 derivatives.

Single colonies of each strain were inoculated into minimal medium (5 mls) containing 1.5% glucose, 0.4% NZ-amine, 10mM MgSO₄, 1 ug/ml each FeSO₄ and MnSO₄, and ampicillin, tetracycline and chloramphenicol. These cultures were grown overnight at 37°C, then diluted 1:12 into the same medium plus 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) and 0.5% w/v ¹³C-glucose (labelled in the C6 position). This culture was transferred to an NMR tube and used directly for NMR spectroscopy at 28°C using a Varion Associates XL200 NMR spectrometry system. Aeration of the culture was achieved by rotating the tube and pumping air through the culture during the run. Spectra were acquired at 3-hour intervals.

Analysis of results

Table II summarizes the efficiency of conversion of glucose to tryptophan by the strains described immediately above. From the intensity of the peaks of the NMR spectra corresponding to C6-glucose and C6-tryptophan, one can calculate the increase in tryptophan relative to the decrease in glucose (after making allowance for the molecular weights of the two compounds).

The results suggest that the pgi block in D2705 serves to substantially increase efficiency of conversion of glucose to tryptophan, when compared to D2704.

TABLE II
Efficiency of Conversion of Glucose to Tryptophan

Strain	C6 Intensity		Conversion* (%)
	Glucose	Tryptophan	
D2704 (Pgi ⁺) pD2625 pD2634 pD2643	-380	+16.4	4.9
D2705 (Pgi ⁻) pD2625 pD2634 pD2643	-185	+18.6	11.4

*Correction factor 1.133 applied for molecular weight difference

Effect of Chromosomal Mutations on Tryptophan Production

Twenty milliliter cultures of the strains listed in Table III below were grown in side-arm flasks at 30°C on a rotary shaker at 280 rpm. The growth medium was MINIMAL MEDIUM #3 described hereinabove supplemented with 1% glucose and 0.4% casamino acids (Difco). Culture supernatants were assayed for tryptophan after 3 days, by the method of Spies and Chamber (Anal. Chem. 20:30, 1947).

25

TABLE III

Strain	Relevant Chromosomal Allele *				TRP (ppm)
	<u>tyrA</u>	<u>tyrR</u>	<u>trpE</u>	<u>aroG</u>	
D2322	w.t.	w.t.	w.t.	w.t.	2
D2321	4	w.t.	w.t.	w.t.	2
D2320	4	366	w.t.	w.t.	3
D2325	4	366	FBR	w.t.	44
D2326	w.t.	366	FBR	w.t.	7
D2402	4	366	FBR	FBR	91

w.t. = wild type;

* all strains are derivatives of W3110F⁻ and carry, in addition, the following markers: (argF-lac) U169, tna 2, bglR6, (lysC-pgi-malE), trpR, (anthranilate-resistance)

Effect of Transaminase A activity on Tryptophan Accumulation

Cultures used in Table IV were grown and assayed exactly as described in the legend to Table III except that the growth medium was MINIMAL MEDIUM supplemented with 2% glucose, 1% NZ-amine, 1mM IPTG, chloramphenicol (20 mg per ml) and ampicillin (100 mg per ml).

TABLE IV

Strain	Relevant Chromosomal Alleles **	TRP (ppm)
D2432 (pD2333,pDM136)	(<u>tyrB</u> ⁺)	66
D2550 (pD2333,pDM136)	<u>tyrB507</u> , <u>zjb::Tn10</u>	473

** These strains also carry the following markers: (argF-lac) U169, aroG^{FBR}, Ø(trp-lac) W205, trpE^{fbr31}, tyrR366, tyrA4, tna2, bglR6, (lysC-pgi-malE), trpR, (anthranilate-resistance).

Effect of Plasmid Genes on Tryptophan Production

5 Fifty milliliter cultures of the strains listed in Table V below were grown in 500 ml baffled flasks, in triplicate, for 44 hours on a rotary shaker (280 rpm) at 30°C. The growth medium was MINIMAL MEDIUM supplemented with 5% glucose, 1% NZ-amine, 1mM IPTG, tetracycline (5mg/ml), ampicillin (100 mg/ml) and chloramphenicol (20 mg/ml). Culture

supernatants were assayed by the method of Spies and Chamber (Anal. Chem. 20: 30, 1947). The genotype of the host strain, D2618, is as follows:
 W3110 F-(argF-lac) U169, aroG^{FBR}, ϕ (trp-lac) W205, trpE^{FBR31},
tyrR366, tyrA4, tna2, bglR6, (lysC-pgi-malE) , tyrB507, zjb, trpR,
 5 (anthranilate-resistance).

TABLE V

Host Strain	Plasmids Carried				Relevant Plasmid Genes					TRP (ppm)
	pM136	pD2623	pD2434	pD2625	<u>trpA-D</u>	<u>trpE^{FBR}</u>	<u>aroG^{FBR}</u>	<u>serA</u>	<u>lacI</u>	
D2618	+		+		+		+		+	430 \pm 60
D2618		+	+		+	+	+		+	910 \pm 60
D2618		+		+	+	+	+	+	+	1490 \pm 20

Tryptophan Production in Serine-Deaminase Deficient Strain

Cultures indicated in Table VI were grown and assayed exactly as described in the legend to Table V above except that the incubation period was 48 hours and each value is the average of 2 flasks only. D2715 is derived from D2618 by introducing deletions into the pheA gene and into the
 10 structural gene for serine deaminase.

TABLE VI

Strain	TRP (ppm)
D2618 (pD2623, pD2625)	2000
D2715 (pD2643, pD2625, pD2634)	2400

Deposited Microorganisms

The following microorganisms have been deposited in the American Type Culture Collection.

<u>Strain</u>	<u>Plasmids</u>	<u>ATCC Accession Number</u>
D2715	pD2643, pD2625, pD2634	39796
C534	pC501	39794
JC158	pD2537, pC520	39797
C534	pD2310, pDM136	39795
MEW191		39575
1K15-5		39576
D2715	pD2625, pD2634, pD2643, pGM3207	53064

Strain D2715 in which are cloned pD2625, pD2634, pD2643 and pGM3207 is not only deposited under ATCC Accession Number 53064 but is also deposited in a security deposit to which the United States Patent and Trademark Office has irrevocable access. (Security Deposit No. 799.)

- 5 It will be readily apparent that the inventor has provided, in addition to the unique bacterial hosts and plasmids, a method for increasing the production of tryptophan in a host of the strains of Escherichia, particularly E. coli. The general method according to the invention comprises the steps of transforming an E. coli host deficient in tryptophan-
- 10 ase with plasmid-borne genetic information to control tryptophan production divided between at least two plasmids, growing the transformed host in an appropriate nutrient medium for a period of time sufficient to produce tryptophan as exemplified above, and producing tryptophan by the transformed host. Tryptophan from the culture broth may be removed by any
- 15 conventional technique. The tryptophan so obtained may be further refined or purified as desired. Preferably the host, in addition to being Tna^- , will be deficient in the activity of phosphoglucose isomerase (pgi^-). The host, in addition to or instead of being pgi^- , may also have DAHP synthetase activity resistant to aromatic amino acid inhibition, anthran-
- 20 ilate synthetase activity resistant to tryptophan inhibition, deficiencies in the activity of lac repressor, aromatic transaminase A, L-serine deaminase either singly or in any combinations thereof.

- The inventor has also provided a method for increasing the production of tryptophan in a host of the genus Escherichia, particularly
- 25 E. coli by transforming an E. coli host deficient in the enzyme

tryptophanase with a plasmid carrying plasmid-borne genetic information to control tryptophan production, a portion of said information including lac operator/promoter control, growing the transformed host in an appropriate medium as exemplified above and producing tryptophan by the transformed
5 host.

Preferably the E. coli host will have the additional characteristics described above with respect to pgi⁻, DAHP synthetase, anthranilate synthesis, lac repressor activity, aromatic transaminase and L-serine deaminase.

10 In a most preferred embodiment, the preferred E. coli host is transformed with plasmid-borne genetic information to control tryptophan production divided between at least two plasmids, a portion of the information to control tryptophan production being under lac operator/promoter control.

15 The foregoing description of the invention is intended to provide a guide, to those ordinarily skilled in the arts to which the invention pertains, to the means to attain and practice the invention as claimed hereinbelow. It will be readily apparent to those ordinarily skilled in the art to which the invention pertains that one may depart
20 from the exact description of the invention contained herein without departing from the scope of the invention as claimed.

WHAT IS CLAIMED IS:

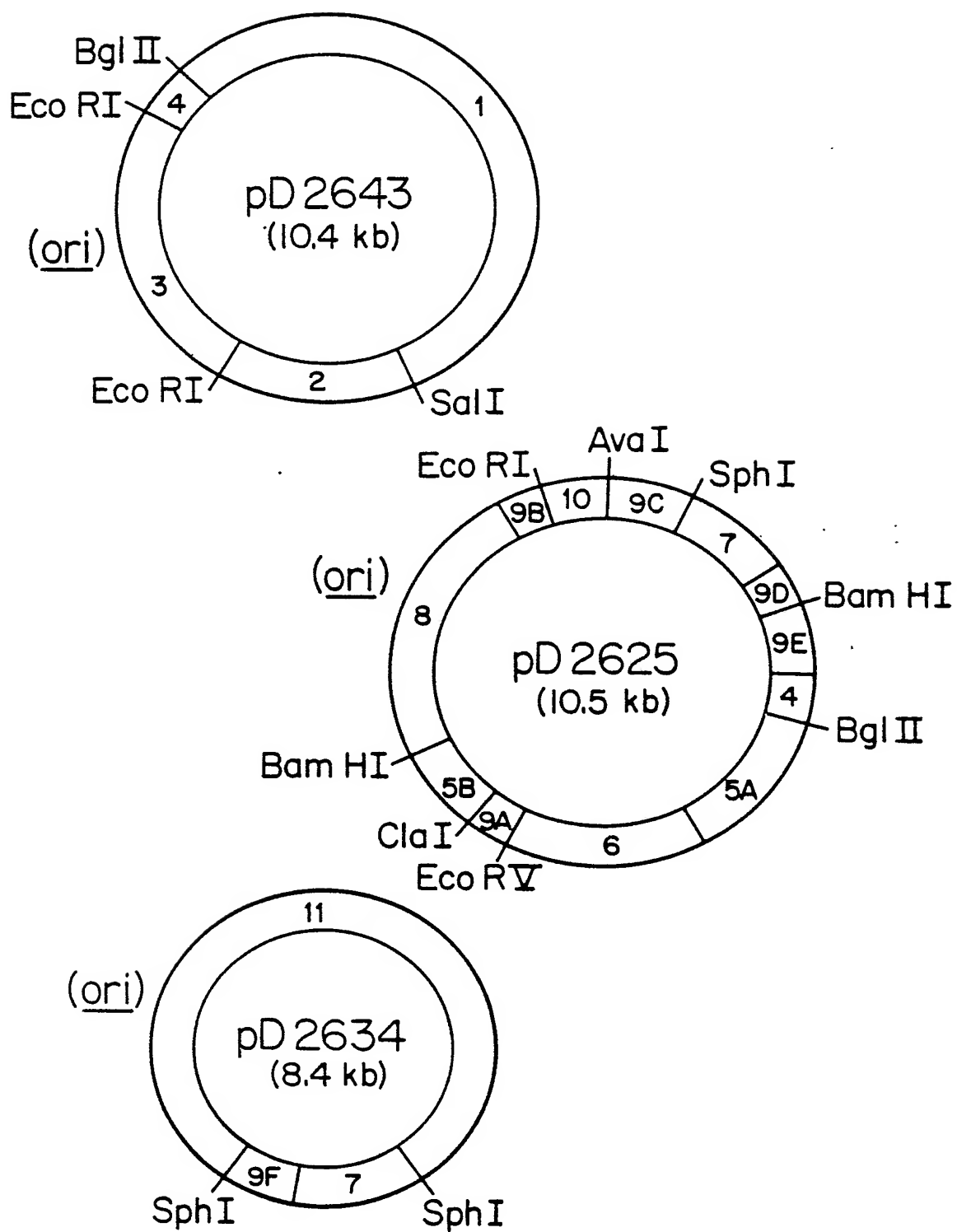
1. A bacterium comprising a host of the genus Escherichia deficient in the enzyme tryptophanase carrying plasmid-borne genetic information to control tryptophan production divided between at least two replication-compatible plasmids.
- 5 2. A bacterium comprising a host of the genus Escherichia deficient in the enzyme tryptophanase carrying plasmid-borne genetic information to control tryptophan production divided between at least two plasmids, a portion of said information under lac operator/promoter control.
- 10 3. The bacterium of Claim 2 having information to control serine biosynthesis including serA, serB and/or serC not under lac operator/promoter control.
4. The bacterium of Claim 1 wherein at least one of said plasmids carries information controlling biosynthesis of the common
15 precursors of the aromatic amino acids.
5. The bacterium of Claim 4 wherein at least one of said plasmids carries genetic information for DAHP synthetase activity resistant to inhibition by aromatic amino acids under lac promoter control.
- 20 6. The bacterium of Claim 2 wherein said plasmid borne genetic information for control of tryptophan production includes trpA-E genes wherein trpA-D are under lac operator/promoter control, said plasmid carries a deletion in the trp attenuator region and wherein trpE codes for anthranilate synthetase resistant to inhibition by tryptophan.
- 25 7. The bacterium of Claim 2 wherein said plasmid-borne genetic information further includes a region for constitutively produced lac repressor activity.

8. The bacterium of Claim 2 wherein said host additionally has activities selected from the group consisting of DAHP synthetase resistant to aromatic amino acid inhibition, anthranilate synthetase resistant to tryptophan inhibition, deficiency in activity of: lac repressor; phospho-
5 glucose isomerase; aromatic transaminase A; L-serine deaminase; tyrA; pheA; trpR and combinations thereof.

9. A method for the production of tryptophan comprising growing the bacterium of Claim 1 in an appropriate culture medium for a period of time suitable to produce tryptophan, and removing said tryptophan from the
10 culture medium.

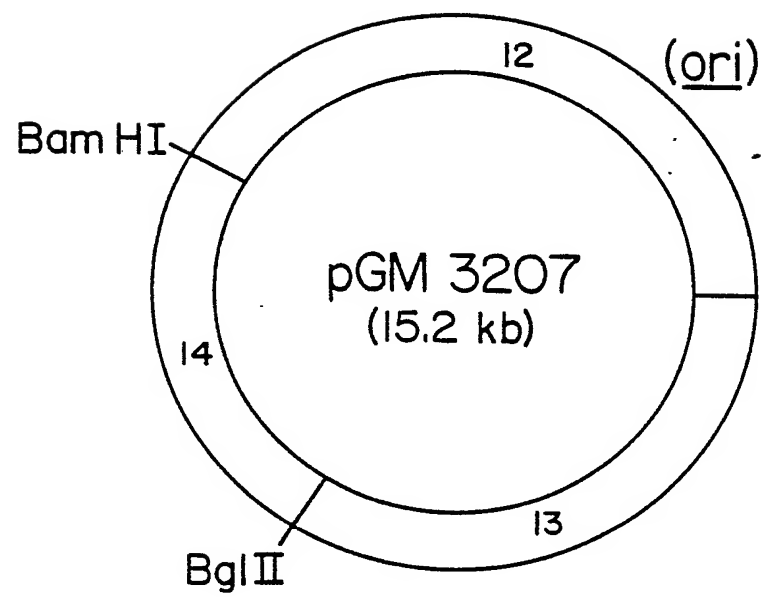
10. A method for increasing the production of tryptophan by an E. coli bacterium comprising transforming the host of Claim 1 with at least two plasmids, said plasmids being divided therebetween genetic information to control tryptophan production, and growing said transformed
15 host in culture medium appropriate for tryptophan production by said transformed host.

Figure 1



2 / 2

Figure 2



INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US85/01542**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="text-align: center; font-family: monospace; font-size: 1.2em;"> C 12 N 15/ 00 435/172.3 </div>																										
II. FIELDS SEARCHED <div style="text-align: center; font-size: 0.8em;">Minimum Documentation Searched ⁴</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; text-align: left; padding: 5px;">Classification System</th> <th style="text-align: left; padding: 5px;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px; vertical-align: top;">U.S.</td> <td style="padding: 5px; vertical-align: top;">435/108,172.3,253,317 935/29,40,41,60,73</td> </tr> </table> <div style="text-align: center; font-size: 0.8em; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵</div> <div style="text-align: center; padding: 5px;"> CHEMICAL ABSTRACTS DATA BASE 1980-1985 BIOSIS DATA BASE 1969-1985 </div>			Classification System	Classification Symbols	U.S.	435/108,172.3,253,317 935/29,40,41,60,73																				
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; text-align: left; padding: 5px;">Category *</th> <th style="text-align: left; padding: 5px;">Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷</th> <th style="width: 15%; text-align: left; padding: 5px;">Relevant to Claim No. ¹⁸</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">U.S., A 4,371,614, Published 01 February 1983, Anderson et al.</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">N, AIBA, et al., <u>APPLIED AND ENVIRONMENT MICROBIOLOGY</u>, Vol 43, No. 2, p. 289-297, 1982.</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">N, BARTH, et al, <u>J. of BACTERIOLOGY</u>, Vol 135, No. 3, p. 760-765, 1978.</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">EPO 0077 196, PUBLISHED 20 APRIL 1983, GENEX CORPORATION</td> <td style="vertical-align: top; padding: 5px;">4, 5 and 8</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">N, FARABAUGH, <u>NATURE</u>, VOL 274, p 765-769, 1978</td> <td style="vertical-align: top; padding: 5px;">7 and 8</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">N, ROBERTS, IN <u>PROMOTERS, STRUCTURES AND FUNCTION</u>, PRAGER PUBLISHERS, NY, NY, (RODRIGUEZ AND CHAMBERLIN, Eds), p 452-461, 1982</td> <td style="vertical-align: top; padding: 5px;">2, 3 and 5-8</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">N, TRIBE, et al, <u>APPLIED AND ENVIRONMENTAL MICROBIOLOGY</u>, Vol 38, No 2, p 181-190, 1979</td> <td style="vertical-align: top; padding: 5px;">1-10</td> </tr> </table> <div style="font-size: 0.8em; margin-top: 10px;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> </div>			Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	Y	U.S., A 4,371,614, Published 01 February 1983, Anderson et al.	1-10	Y	N, AIBA, et al., <u>APPLIED AND ENVIRONMENT MICROBIOLOGY</u> , Vol 43, No. 2, p. 289-297, 1982.	1-10	Y	N, BARTH, et al, <u>J. of BACTERIOLOGY</u> , Vol 135, No. 3, p. 760-765, 1978.	1-10	Y	EPO 0077 196, PUBLISHED 20 APRIL 1983, GENEX CORPORATION	4, 5 and 8	Y	N, FARABAUGH, <u>NATURE</u> , VOL 274, p 765-769, 1978	7 and 8	Y	N, ROBERTS, IN <u>PROMOTERS, STRUCTURES AND FUNCTION</u> , PRAGER PUBLISHERS, NY, NY, (RODRIGUEZ AND CHAMBERLIN, Eds), p 452-461, 1982	2, 3 and 5-8	A	N, TRIBE, et al, <u>APPLIED AND ENVIRONMENTAL MICROBIOLOGY</u> , Vol 38, No 2, p 181-190, 1979	1-10
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IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search ² <div style="font-family: cursive; font-size: 1.2em; margin-top: 10px;">November 4, 1985</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report ³ <div style="font-size: 1.2em; margin-top: 10px;">15 NOV 1985</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority ¹ <div style="text-align: center; margin-top: 10px;">ISA/US</div> </td> <td style="padding: 5px;"> Signature of Authorized Officer ²⁰ <div style="text-align: center; margin-top: 10px;">J.A. Huleatt </div> </td> </tr> </table>			Date of the Actual Completion of the International Search ² <div style="font-family: cursive; font-size: 1.2em; margin-top: 10px;">November 4, 1985</div>	Date of Mailing of this International Search Report ³ <div style="font-size: 1.2em; margin-top: 10px;">15 NOV 1985</div>	International Searching Authority ¹ <div style="text-align: center; margin-top: 10px;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center; margin-top: 10px;">J.A. Huleatt </div>																				
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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A,	N, GIBSON, et al, <u>BACTERIOLOGICAL REVIEWS</u> , Vol. 32, No. 4, Pt. 2, p 465-492, 1968	1-10
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.